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ESSENTIAL GUIDES TO METHOD DEVELOPMENT IN CAPILLARY ELECTROPHORESIS

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Introduction

Capillary electrophoresis encompasses a number of related separation approaches, some of which are adapted to the requirements of specific applications (**Figure 1**). They share in common the use of electrolyte solutions as mobile phase, the use of capillary tubes as the separation column, and the use of an electric field to induce sample and mobile phase transport. This allows a similar instrument platform to service all capillary electrophoretic separation techniques with only minor modifications for specific applications. Detection is usually by UV-visible absorption through the fused silica capillary wall, or occasionally by fluorescence, electrochemical or mass spectrometric detection. Contemporary instruments are also highly automated for ease of use and improved control of critical experimental variables.

Classification of capillary electrophoretic techniques according to their usual applications is given in **Table 1**. These techniques can be considered as general, sample-type specific, in an early development phase, or of minor importance. Such a broad range of descriptive terms requires further elaboration to indicate how we propose to treat these techniques in this article. Capillary zone electrophoresis (CZE), or simply capillary electrophoresis, and micellar electrokinetic chromatography (MEKC) are widely used and complementary techniques for the separation of ionic and neutral molecules. They are the most important and general in terms of the number of applications and frequency of use. Capillary electrochromatography (CEC) is a relatively new and promising technique with a range of applications similar to liquid chromatography. Since electro-driven flow has been shown to provide both theoretical and practical advantages over pneumatic-driven flow, it has the potential to become a major separation technique. At present, too little is known about the technique to provide a definitive guide to method development, especially as in the future it is likely that new column materials will be developed specifically for capillary electrochromatography with properties different to those currently used. Capillary gel electrophoresis (CGE) is an important technique for the separation of biopolymers but is little used outside of laboratories that perform this type of analysis. Capillary isoelectric focusing (CIEF) is a specialized technique within the field of macromolecule zwitterion separations,

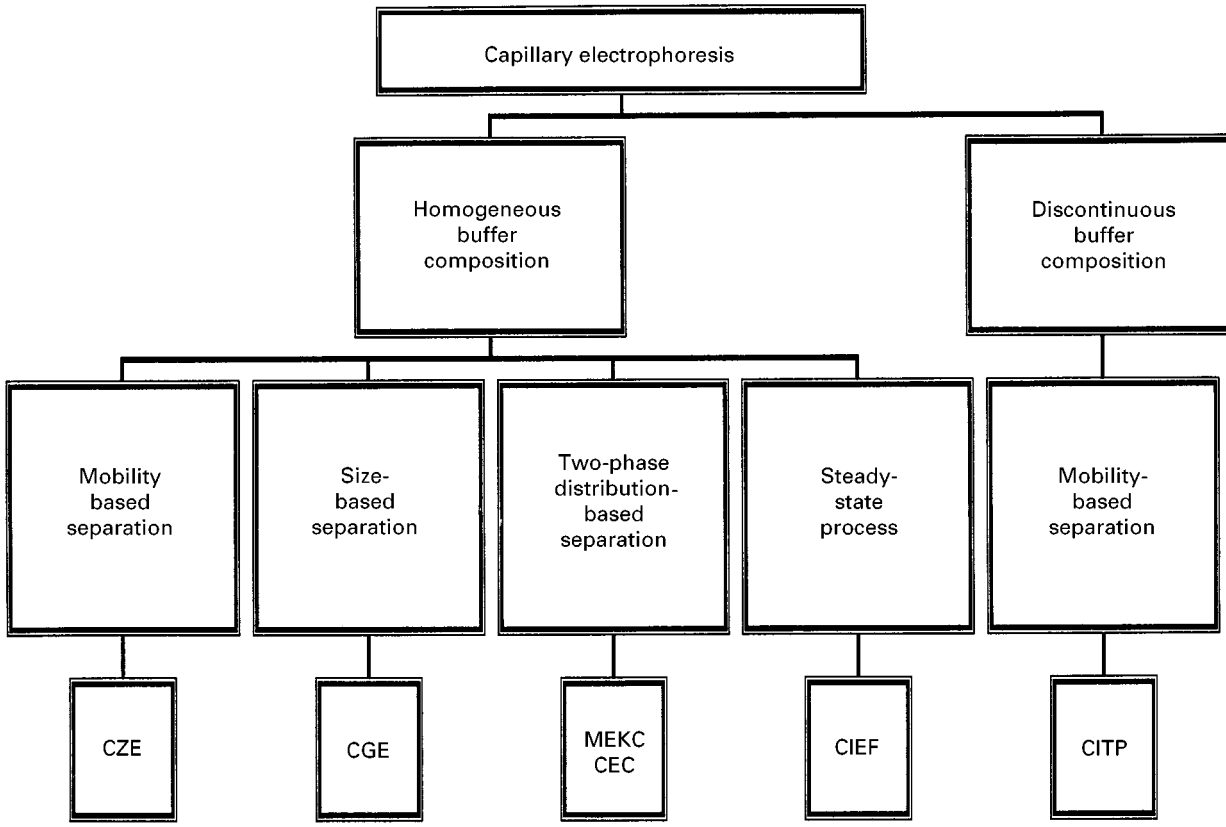

Figure 1 Classification of capillary electrophoretic separation methods based on buffer type and mechanism. CZE = capillary zone electrophoresis; CGE = capillary gel electrophoresis; MEKC = micellar electrokinetic chromatography; CEC = capillary electrochromatography; CIEF = capillary isoelectric focusing; and CITP = capillary isotachophoresis.

largely proteins, requiring special buffers to generate a continuous pH gradient. Capillary isotachophoresis (CITP) is not widely used for separations, it can be rather difficult and tedious to optimize, and yields an integral signal that is different to other separation techniques. Many samples that can be separated by capillary isotachophoresis can also be separated by other electrophoretic techniques more familiar to separation chemists. It is finding increasing use to preconcentrate ions for separation by capillary zone electrophoresis. With this framework in mind we propose to provide general guidelines for method development in capillary zone electrophoresis, micellar electrokinetic chromatography, and gel electrophoresis with only comments and brief instructions applicable to the other capillary electrophoretic techniques.

Sample Suitability

Table 1 provides a general guide to method selection by analogy to established applications. For biopolymers capillary electrophoretic techniques often select themselves, for other compounds the capillary electrophoretic techniques have to be considered in terms of suitability drawn against other existing chromatographic methods. Reasonable solubility in aqueous solution is required for most separation modes. Non-aqueous capillary electrophoresis is little developed (although promising) and techniques such as micellar electrokinetic chromatography can separate hydrophobic compounds but provide little selectivity. Gas chromatography is usually a better choice for the separation of volatile hydrophobic compounds. High pressure liquid chromatography is often a better choice when low level detection, structural elucidation by mass spectrometry or preparative-scale separations are required. The concentration sensitivity of the capillary electrophoretic techniques using UV-visible absorption detection is limited by the small cross column pathlength and small injection volumes to solutions containing at least 1–10 $\mu g\ mL^{-1}$ and for ease of operation 0.1 $mg\ mL^{-1}$ or above is preferred. Various stacking and preconcentration techniques may improve detection limits but these require additional effort and time for optimization that may not be justifiable if another technique is suitable for the separation. Within these

Table 1 Common separation methods using capillary electrophoretic techniques

Technique	*Separation mechanism*	*Applications*
Zone electrophoresis	Differences in charge-to-size ratios	Inorganic and organic ions Ionizable compounds Zwitterions Biopolymers
Micellar electrokinetic chromatography	Distribution of neutral and partially ionized compounds between charged micelles and electrolyte solution	Water-soluble neutral compounds Weak acids and bases
Gel electrophoresis	Differences in size and charge (but not size-to-charge ratio) by migration through a gel matrix or entangled polymer network with a range of pore sizes	DNA fragments SDS proteins Macromolecules
Electrochromatography	Distribution between a solid stationary phase and mobile electrolyte solution	Neutral compounds Weak acids and bases Ions
Isoelectric focusing	Differences in isoelectric points in a continuous pH gradient	Proteins Zwitterionic compounds
Isotachophoresis	Differences in electrophoretic mobility of ions sandwiched between two buffers containing ions of greater (leading) and lower (trailing) mobility	Preconcentration of ions

restrictions it is obvious that many sample types and problems can be handled by capillary electrophoretic techniques accounting for its expanding use in analytical chemistry.

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Selecting System Variables

Virtually all separations are carried out in fused silica capillary columns 50–100 μm internal diameter and up to 1-m long. Large-bore capillaries provide greater loading capacity and a higher detector response because of the longer pathlength (on-column detection) but generate larger currents and are less efficient at heat dissipation. Small-diameter columns show increased adsorption character due to their larger inner surface area-to-volume ratio but provide more efficient heat dissipation. If detection limits are not a problem, then a small inner diameter column should be used. The choice of capillary length is a compromise between speed (short columns) and separation capacity (long columns). Unless the separation is unusually complicated capillaries should be short (25–50 cm). When a new capillary is put into use or is suspected of being contaminated, a conditioning procedure is required. Washing with a solution of sodium hydroxide, water, and buffer as indicated in **Table 2** is normally sufficient. Capillaries with an interior coating are used to alter electroosmotic flow or to minimize analyte adsorption by the capillary wall, particularly for macromolecules. Electroosmotic flow is optimized to obtain useful separations in MEKC and CEC, is often used to improve separations and total sample detection for ions of opposite charge in CZE, but is usually undesirable in CGE, CIEF and CITP. So it is in the later techniques that capillaries with chemically bonded or physically adsorbed coatings are used.

Separations are usually performed with a voltage of 10–30 kV. High voltages provide faster separations with higher efficiency provided that the heat generated is effectively dissipated. A plot of current against applied voltage can be used to optimize operating conditions. The fastest and most efficient separations are obtained at the upper end of the linear portion of the plot. A positive deviation in the plot indicates that the heat removal capacity of the system is being exceeded. Capillary electrophoretic separations are usually performed at or close to room temperature (25°C). Temperature control, however, is important and separation capillaries are thermostated in an air or liquid bath. Thermostating is used to remove heat and to establish a constant temperature. Poor thermostating results in lower efficiency and poor reproducibility of migration times. Temperature is a useful operating variable, which can be used to modify migration times and selectivity, but is generally considered only suitable for fine tuning

Table 2 A guide for selecting initial conditions in capillary electrophoretic separations

Parameter	*Setting*
Column	Initial experiments use a fused silica capillary 30–50-cm long and 50- or 75-μm internal diameter. Short columns are appropriate for trial experiments. The complexity of the sample dictates the length. For 2–10 analytes use 35–40 cm; 11–50 analytes 50–60 cm; 50–80 analytes 70–80 cm; and > 80 analytes 90–100 cm. Smaller diameter columns (25 or 50 μm) provide higher efficiency but lower sample loading capacity.
Initial conditioning	Rinse with 0.1 M sodium hydroxide for 30 min. Flush with water for 15 min followed by the separation buffer for 15 min.
Voltage	Usual range is 10–30 kV. High voltages provide faster separations and greater separation efficiency. The method employed to dissipate heat, the column internal diameter, and buffer type and concentration all affect this decision. Use the highest voltage that does not exceed 100 μA current as a rough guide. Otherwise plot current against voltage (2.5-kV increments) and operate at a voltage towards the upper portion of the linear plot.
Temperature	Initial experiments use 20–25°C. Selectivity and separation speed varies with temperature, which is optimized to fine-tune a separation (vary from 20 to 60°C in 5°C increments).
Injection	Hydrodynamic (e.g. 3 s at 0.5 p.s.i.) or electrokinetic (2–5 nL)
Detection	Absorption maximum of the analyte of interest, for which the weakest signal is expected because of low concentration or low absorbance. If analyte detection properties are unknown try 200–230 nm.

nearly acceptable separations. Subambient temperatures are not commonly used, as they are less convenient and result in poorer kinetic separation properties.

In general, the sample should be prepared such that the analytes of interest are present in a suitable solution, free from interferences, and at an appropriate concentration for detection. The ionic strength of the sample should be no greater than that of the buffer, with a more or less similar pH to the buffer, and free of matrix problems associated with column wall adsorbing materials and particle matter. For the best peak shapes and resolution the concentration of the injected sample should be about 100 times lower than the concentration of the buffer. Syringe filters for particle removal and ion exchange membrane filtration devices to reduce excessive concentrations of common matrix ions are available. Proteins and similar macromolecules, if not of interest to the analysis, should be precipitated prior to separation to minimize column fouling. Analytes of low water solubility may have to be dissolved in a water-miscible organic solvent or mixture of organic solvent and separation buffer. For other samples it is common practice to dissolve the sample in the run buffer, a diluted solution of the run buffer, or water. Samples are introduced into the separation capillary by hydrodynamic or electrokinetic injection. Both methods provide reproducible injection volumes but sampling bias is associated with electrokinetic injection, which injects increasing amounts of sample components in proportion to their mobility. Hydrodynamic injection is not suitable for CEC and CGE because of the high flow resistance of packed columns.

Capillary Zone Electrophoresis

Once the system variables are set within reasonable ranges the parameters that have most effect on migration times and selectivity are the composition, concentration and pH of the run buffer and the presence of additives, if used, to provide additional selectivity optimization. For a good separation by CZE four features are important: (i) the individual mobilities of the analytes must be different; (ii) the background electrolyte must be homogeneous and the field strength uniform along the column; (iii) neither analytes nor matrix components must interact with the column wall; and (iv) the conductivity of the buffer must substantially exceed the total conductivity of the sample components. Suitable common buffer recipes for a wide pH range are given in **Table 3**. Additional buffers with their pK_a and anion mobility values are given in **Table 4**.

Ionic strength and pH greatly affect selectivity and separation time and should be course tuned in initial screening experiments. Low pH is favourable for separating anions (all anions are less mobile) and a high pH is preferred for cation separations. The practical pH range is limited roughly to between 2 and 12. If the pK_a of the sample components is known or can be reasonably estimated, pH optimization should start with a pH $\approx$ pK_a. Weak acids and bases change from the neutral form to the fully ionized form over about 4 pH units. In the neutral form their electrophoretic mobility is zero and they all migrate at a fixed velocity due to the electroosmotic flow in common with all neutral species. When totally ionized the ion moves with a constant electrophoretic velocity and may be separated from other

Table 3 Recipes for preparing some common electrophoretic buffers (100 mL of 60 mM buffer)

pH	*Buffer system*	*Acid*	*Base*
	Phosphate	85% Phosphoric acid	Potassium dihyrogenphosphate
2		395.3 mg	349.9 mg
2.5		205.3 mg	574.3 mg
3.0		81.4 mg	720.5 mg
	Acetate	1.0 M Acetic acid	Sodium acetate
3.5		5.67 mL	26.6 mg
4.0		5.08 mL	75.8 mg
4.5		3.81 mL	174.6 mg
5.0		2.13 mL	317.6 mg
5.5		0.89 mL	419.1 mg
	Phosphate	Sodium dihyrogenphosphate ($1H_2O$)	Disodium hydrogenphosphate ($2H_2O$)
6.0		779.2 mg	61.9 mg
6.5		692.8 mg	174.3 mg
7.0		512.2 mg	407.2 mg
7.5		280.7 mg	705.9 mg
8.0		115.5 mg	919.0 mg
	Borate	Boric acid	Disodium tetraborate ($10H_2O$)
8.0		320.9 mg	77.3 mg
8.5		232.7 mg	213.2 mg
9.0		59.3 mg	480.6 mg
	Borate	Disodium tetraborate ($10H_2O$)	0.1 M Sodium hydroxide
9.5		371.0 mg	41.77 mL
10.0		371.0 mg	52.72 mL

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ions based on differences in their charge-to-size ratio. When partially ionized the ions migrate with an effective mobility that changes between the two extreme values in a sigmoid fashion as the pH is varied (**Figure 2**). Ions may be separated in their fully ionized form or partial ionized form as a matter of circumstance; that is, at those conditions that maximizes the difference in charge-to-size ratios. Because changes in mobility tend to be large for partially ionized solutes small pH changes (0.1–0.5 pH units, or smaller for complex mixtures) are used to optimize the separation.

If the pK_a values for a sample are unknown, conduct initial separations in a series of buffers at or near pH 2.5, 4.0, 5.5, 7.0, 8.5 and 10 (see Table 3 for appropriate buffers). To obtain reproducible results over the pH range 4 to 7, careful column conditioning is important. From the plot of the effective mobility against pH identify the most promising pH range for the separation. Optimization then proceeds in smaller changes in pH units as before.

To optimize the buffer concentration initial experiments are performed with a concentration of 30–100 mM for 50-μm internal diameter columns and 20–50 mM with 75-μm internal diameter columns. Lower ionic strength buffers are used to obtain faster separations, when selectively is high, and to separate simple mixtures containing a few analytes. Higher ionic strength buffers are used for the separation of complex mixtures or to separate analytes with small differences in their electrophoretic mobility. If stacking is used to enhance analyte detectability then the difference in ionic strength between the buffer (high ionic strength) and the sample should be maximized. From Table 4 inorganic buffers are likely to provide better peak shapes for high mobility ions and Good-type (zwitterionic) buffers for low mobility ions. Zwitterionic buffers are useful for many applications where a high concentration and buffering capacity is desirable because of their low specific conductivity, which allows more favourable kinetic separation conditions to be employed.

For difficult separations the selectivity can be further modified by employing secondary chemical equilibria and solvation effects by adding appropriate reagents or solvents to the electrolyte system (**Table 5**). Increasing the ionic strength of the electrolyte by adding salts such as potassium sulfate modifies the charge and/or conformation of proteins and reduces wall interactions. Metal cations such as Cu^{2+}, Zn^{2+}, Ca^{2+} coordinate to proteins and peptides modifying the net charge. Also, alkanesulfonic acids bind selectively to proteins and peptides through hydrophobic interactions modifying the surface charge as well as reducing wall interactions. Slow

Table 4 Suitable buffers for capillary electrophoresis. Mobility values are at zero ionic strength and 25°C (in 10^{-9} m^2 V^{-1} s^{-1})

Buffer	*pK_a*	*Mobility*
Phosphoric acid	2.12 (pK_1)	− 35.10
	7.21 (pK_2)	− 58.30
	12.32 (pK_3)	− 71.50
Malonic acid	2.90 (pK_1)	
	5.70 (pK_2)	
Citric acid	3.13 (pK_1)	− 28.70
	4.76 (pK_2)	− 54.30
	6.40 (pK_3)	− 70.80
Lactic acid	3.85	− 35.80
Hydroxyisobutyric acid	3.97	− 33.50
Glutamic acid	4.38	− 28.90
Acetic acid	4.76	− 42.40
MES [2-(*N*-morpholine)ethanesulfonic acid]	6.13	− 26.80
MOPS [3-(*N*-morpholine)propanesulfonic acid]	7.20	− 24.40
MOPSO [2-hydroxy-4-morpholinepropanesulfonic acid]	6.79	− 23.80
ACES [*N*-2-acetamido-2-aminoethanesulfonic acid]	6.84	− 31.30
Imidazole	7.17	52.00
BES [2-(bis{2-hydroxyethyl}amino)ethanesulfonic acid]	7.16	− 24.00
HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]	7.51	− 21.80
TRICINE [*N*-{tris(hydroxymethyl)methyl}glycine]	8.15	
TRIS [tris(hydroxymethyl)aminoethane]	8.08	29.50
TAPS [3-{tris(hydroxymethyl)methyl}aminopropanesulfonic acid]	8.30	− 25.00
BICINE [*N,N*-bis(2-hydroxyethyl)glycine]	8.35	
Glycylglycine	8.40	
Ammonia	9.26	
Ethanolamine	9.50	44.3
CHES [2-(cyclohexylamino)ethanesulfonic acid]	9.50	
Triethylamine	9.87	
CAPS [3-(Cyclohexylamino)propanesulfonic acid]	10.40	
Diethylammonium	11.40	37.9

adsorption/desorption interactions with the column wall cause peak broadening and tailing and irreversible adsorption leads to modification of the capillary wall. These problems are caused by electrostatic or hydrophobic interactions between macromolecules (usually) and the column wall. Solutions to this problem include using extreme pH buffers, high ionic strength electrolytes, and by using dynamic or chemically bonded wall-coated capillaries. There are no universal solutions and effective methods have to be tailored to the properties of the analyte. Buffer additives are usually used at concentrations of 5–60 mM except for modification of the ionic strength of the electrolyte where much higher concentrations are often required (e.g. 50–250 mM). Urea, which forms hydrogen-bond complexes with proteins and peptides, but is nonionic, is often used at concentrations of 7 M. The separation of metal cations (alkaline earths, transition metals and lanthanides) is difficult because of their similar ionic conductance. In this case complexing agents, such as α-hydroxyisobutyric acid or citrate are required. Since many cations lack a chromophore complexation is an effective method of introducing a chromophore for convenient detection. There is now considerable literature on the separation of anions by capillary electrophoresis. For fast separations it is necessary to reverse the direction of the electroosmotic flow by adding cationic surfactants below their critical micelle concentration to the buffer

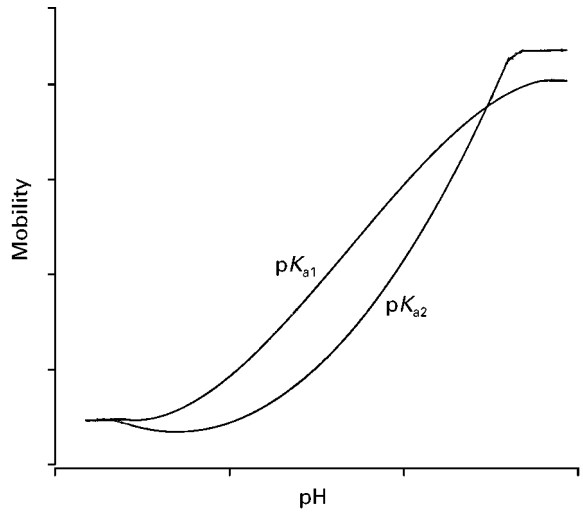

Figure 2 Separation of two hypothetical weak acids as a function of pH by capillary zone electrophoresis.

Table 5 Secondary equilibria used to optimize selectivity in capillary electrophoresis

Additives	*Function*
General considerations	
Inorganic salts	Minimize wall interactions, induce protein conformation changes
Crown ethers	Modify mobility by selective formation of inclusion complexes
Organic solvents	Modify electroosmotic flow, increase solubility of organic ions, modify ion solvation, reduce wall interactions
Urea	Modifies the mobility of proteins by hydrogen-bond complexation
Metal ions	Modify mobility of anions and electroosmotic flow
Alkanesulfonic acids	Modify mobility by ion pair formation, wall adsorption leads to changes in surface properties
Cellulose polymers	Mask active sites on the capillary wall, modify electroosmotic flow
Cationic surfactants	Use to reverse the polarity of the fused silica capillary wall
Organic acids	Modify mobility by ion pair formation
Ion complexation	
Chelate formation (metals)	Polycarboxylic acids (lactate, tartrate, hydroxyisobutyric acid)
	Ethylene-1,2-diaminetetraacetic acid
	Dihydroxyazobenzene-5, 5′-disulfonate
Ion pairing	Ionic surfactants (<critical micelle concentration)
	Cetyltrimethylammonium bromide, tetradecyltrimethylammonium bromide
	Polyvalent metal cations (Ca^{2+}, Al^{3+}, etc.)
	CHES and other alkanesulfonic acids, perchlorate
Ion inclusion	Crown ethers (15-crown-6, 18-crown-6, etc.)
Solvent effects	
Organic solvents	Acetonitrile, methanol, 2-propanol, tetrahydrofuran, etc.
Electrolyte	Ionic strength, concentration of the probe (co-ion)

system. The electroosmotic flow and electrophoretic migration now occur in the same direction. For difficult to separate anions normal (counterflow) operation may be the better option at the expense of longer separation times. To reduce peak broadening the mobility of the sample anions should be matched to those of the background electrolyte. For UV-visible detection indirect detection is frequently employed. This requires the addition of a probe (co-ion) of high molar absorption, in low concentration, with the same charge as the analytes. Examples include chromate (most popular), benzoate, salicylate, phthalate, etc.

Micellar Electrokinetic Chromatography

The addition of an ionic surfactant above its critical micelle concentration to the buffer provides an additional separation mechanism based on distribution of the analytes between the micelles and electrolyte. The velocity with which the micelles migrate to the detector is usually different to the velocity of the bulk electrolyte allowing separations based purely on differences in the analyte distribution constants for neutral compounds. For ions differences in both distribution constants and electrophoretic mobility are important. An acceptable separation also requires favourable kinetic properties (efficiency), provision of an adequate migration window (peak capacity) and a reasonable total separation time. Normally, the experimental conditions are set to establish an acceptable separation time and migration window under conditions where the efficiency is not compromised and the outcome of the experiment controlled by selectivity optimization. Selectivity is influenced largely by the identity of the surfactant and the addition of complexing agents and/or organic solvents to the buffer.

Some common surfactants and their relative solvation properties are summarized in **Table 6**. Method development usually begins with sodium dodecyl sulfate because of its favourable kinetic and chromatographic properties. (**Table 7**). Other surfactants are selected based on their complementary properties to sodium dodecyl sulfate using the system constants of the solvation parameter model as a guide (Table 6). For example, sodium cholate (representative of the bile salts) is a stronger hydrogen-bond base and weaker hydrogen-bond acid than sodium dodecyl sulfate. By similar reasoning a working list of surfactants for selectivity optimization would include sodium dodecyl sulfate, sodium cholate, lithium perfluorooctanesulfonate, sodium *N*-dodeconyl-*N*-methyltaurine and tetradecyltrimethylammonium bromide. Table 6 also provides a framework to identify new surfactants

Table 6 Characteristic properties of common surfactants for micellar electrokinetic chromatography

Surfactant	*Critical micelle concentration (mM)*	*Aggregation number*	*Solvation parameter model system constants**				
			m	*r*	*s*	*a*	*b*
Sodium dodecyl sulfate	8.2	62	2.99	0.46	− 0.44	− 0.30	− 1.88
Tris(hydroxymethyl)aminoethane dodecyl sulfate			2.56	0.57	− 0.66	− 0.33	− 1.56
Sodium dodecyl sulfonate	9.8	54	2.51	0.51	− 0.70	− 0.14	− 1.51
Sodium cholate	13–15	2–4	2.45	0.63	− 0.47	0	− 2.29
Sodium taurocholate	2.8	4	2.43	0.60	− 0.34	0	− 2.06
Sodium deoxycholate	4–6	4	2.67	0.66	− 0.47	0	− 2.47
Sodium taurodeoxycholate	2–4	8	2.62	0.67	− 0.45	0	− 2.17
Sodium *N*-dodecanoyl-*N*-methyltaurine	8.7		3.07	0.72	− 0.50	0.22	− 2.58
Lithium perfluorooctanesulfonate			2.30	− 0.52	0.34	− 0.82	− 0.53
Tetradecyltrimethylammonium bromide	4.4	64	2.82	0.36	− 0.29	0.90	− 2.67
Hexadecyltrimethylammonium bromide	0.026	169	3.40	0.61	− 0.55	0.58	− 3.08
Microemulsion**			3.05	0.28	− 0.69	− 0.06	− 2.81

*The *m* system constant is a measure of the difference in cohesive energy and dispersion interactions for the micelles and electrolyte; the *r* system constant the difference in interactions with lone pair electrons; the *s* system constant the difference in interactions of a dipole type; the *a* and *b* system constants the difference in hydrogen-bond base and hydrogen-bond acid interactions, respectively. The sign of the constant indicates whether the interaction favours distribution to the micelles (positive) or electrolyte system (negative). **Microemulsion consisting of 1.4%wt. sodium dodecyl sulfate, 6.49% wt. butan-1-ol and 0.82%wt. heptane.

with complementary properties to those available at present and to avoid unnecessary experiments with surfactants with different structures but nearly identical selectivity properties.

When selectivity optimization using different surfactant types is exhausted further optimization is achieved by the use of additives (see Table 7). For this purpose the common approaches are the use of mixed

Table 7 Starting conditions for method development in micellar electrokinetic chromatography

Parameter	*Setting*
Sample	1–2 mg mL^{-1} dissolved in methanol or water
Column	Fused silica capillary 30–50-cm long with an internal diameter of 50 μm
Initial conditioning	Flush with 0.1 M sodium hydroxide for 3 min and rinse with the run buffer for 5 min. These conditions will have to be varied depending on the previous use (if any) of the column. It is preferable to reserve individual capillaries for each surfactant.
Buffer	20 mM sodium phosphate–sodium tetraborate pH 8 buffer (or see Table 3 for suitable single buffers) containing 50 mM sodium dodecyl sulfate
Voltage	20–25 kV
Temperature	25°C
Injection	50 mbar 1–2 s (hydrodynamic)
Detection	210 nm (or absorption maximum for analyte with lowest absorbance)
Course tuning selectivity	
Surfactant	Choose surfactants of different selectivity (see Table 6) Sodium cholate (72 mM) Sodium *N*-dodecanoyl-*N*-methyltaurine (50 mM) Tetradecyltrimethylammonium bromide (50 mM) with reverse polarity Other suitable surfactants
pH	Optimize migration window and separation time (lower pH to extend and raise pH to lower) for neutral compounds. Weak acids and bases may show significant changes in electrophoretic behaviour
Additives	Mixed surfactants formed with neutral and ionic surfactants. For example, Brij 35 (polyoxyethylene[23] dodecyl ether) 1–25 mM Organic solvents methanol, 2-propanol, acetonitrile, tetrahydrofuran 1–25% (v/v) Higher molecular mass solvents of low water solubility 1–5% (v/v) Complexing additives such as α-, β-, γ-cyclodextrins, hydroxypropyl-β-cyclodextrin and heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin (5–20 mM)
Fine tuning selectivity	
Modify system properties such as column length, temperature, voltage, buffer type and ionic strength. Surfactant concentration changes the phase ratio but has little effect on selectivity	

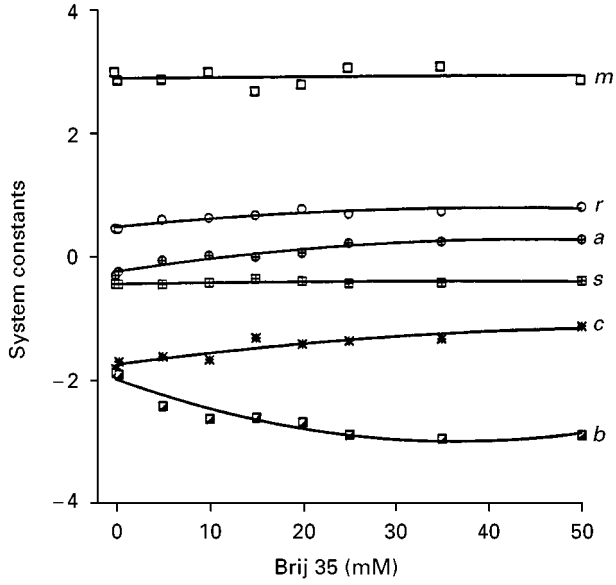

Figure 3 Change in the system constants obtained from the solvation parameter model as a function of the composition of the mixed micelles formed with the neutral surfactant Brij 35 (1–50 mM) and 50 mM sodium dodecyl sulfate. See Table 6. (Reproduced with permission from Poole SK and Poole CF (1997) Variation of selectivity with composition for a mixed-micellar buffer in micellar electrokinetic chromatography. *Journal of High Resolution Chromatography* 20: 174–178.)

surfactant micelles, organic solvents and inclusion complexing agents. A large number of mixed micelles can be employed without any certain prospects of success. Neutral surfactants such as Brij 35 are often chosen first to adjust selectivity and/or the size of the migration window. **Figure 3** shows an example of the use of Brij 35 to change the selectivity of sodium dodecyl sulfate micelles. The solvation properties of the mixed micelles are not changed radically, even at high concentrations of the neutral surfactant, in agreement with predictions made by the interphase retention model. The main change is the gradual decrease in the hydrogen-bond acidity of the mixed micelles, which should provide a useful change of selectivity for the separation of hydrogen-bond bases. Selectivity modification by addition of organic solvent to the buffer is by no means as useful as in reversed-phase liquid chromatography. At low concentrations modifier effects are small and not strongly dependent on solvent identity, and at higher concentrations they lead to deleterious effects on system efficiency and the separation time. By contrast, the use of complexing additives, such as urea and cyclodextrins has to be considered one of the success stories of MEKC for achieving the separation of isomers, enantiomers, and other difficult to separate compounds capable of forming suitable inclusion complexes. **Figure 4** provides an example of the separation of pharmaceutically important estrogens that were only adequately separated in the system containing the complexing additive. The incorporation of low molecular mass organic solvents and cyclodextrins in the micelles is very low. Their main effect on the distribution properties of the system

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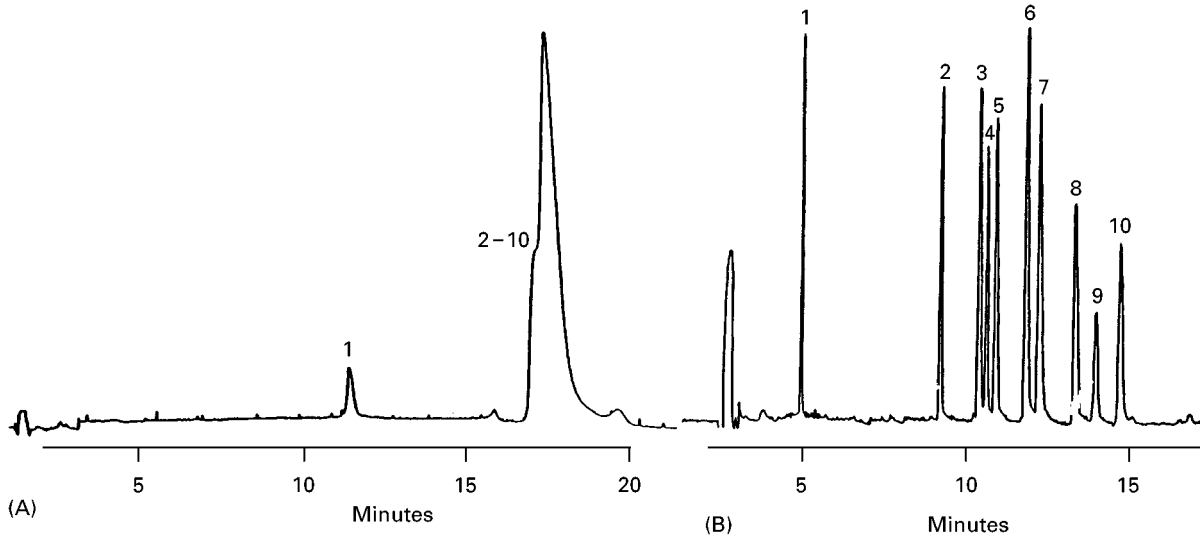

Figure 4 Separation of estrogens by MEKC using a 20 mM sodium phosphate-borate pH 8 buffer containing 50 mM sodium dodecyl sulfate (A) and the same buffer containing 20 mM γ-cyclodextrin (B). Separation conditions: capillary 48.5 cm (effective length 40 cm), internal diameter 50 μm, temperature 25°C, and field strength 20 kV. Compounds: 1 = estriol; 2 = 17β-estradiol; 3 = 17α-estradiol; 4 = 17β-dihydroequilenin; 5 = 17β-dihydroequilenin; 6 = 17α-dihydroequilenin; 7 = 17α-dihydroequilin; 8 = estrone; 9 = equilenin; and 10 = equilin. (Modified from Poole SK and Poole CF (1996) Separation of pharmaceutically important estrogens by micellar electrokinetic chromatography. *Journal of Chromatography A* 749: 247–225, with permission from Elsevier Science.)

is due to changes in the relative solubility of the analytes in the electrolyte.

Capillary Gel Electrophoresis

Capillary gel electrophoresis is used for the separation of macromolecules such as proteins and nucleic acids, whose mass-to-charge ratios do not vary much with size. Separation requires a sieving medium made up of a crosslinked gel or an entangled polymer network. The capillaries are often wall-coated or chemically bonded to minimize electroosmotic flow that tends to destabilize the columns. Columns filled with rigid crosslinked gels, usually polyacrylamide, are characterized by the total amount of monomer and crosslinking agent (%T) and the ratio of crosslinking agent to total amount of monomer and crosslinking agent (%C) used to prepare the column. Larger pore size gels (lower %T) are used for separating DNA sequencing reaction products whereas the narrow-pore media are best for proteins and small oligonucleotides. Entangled polymer networks of linear polyacrylamide, methylcellulose or dextran have the advantage that they can be forced into the capillary as a solution and replaced when needed. Unlike gels, columns are easily prepared in the laboratory and tend to the be more robust. Electrokinetic injection is used for sample introduction. The buffer pH is selected such that the analytes of interest are ionized. TRIS/borate and TRIS/phosphate buffers in the pH range 7.5 to 8.5 (50–200 mM) are fairly general conditions. Sometimes urea (7–8 M) or ethylene glycol (1.5–3.0 M) is added to the buffer as a nonionic denaturing or solubilizing agent and EDTA (about 2 mM) to protect against cation interferences. When SDS-proteins are separated sodium dodecyl sulfate (0.1% w/v) is added to the run buffer. For many practical applications of capillary gel electrophoresis the column materials and reagents required can be purchased in kit form.

Capillary Isoelectric Focusing and Isotachophoresis

Capillary isoelectric focusing is used to separate polypeptides based on differences in their isoelectric points (p*I*) in wall-coated fused silica capillaries to eliminate electroosmotic flow and nonspecific adsorption of the sample with the capillary wall. The capillary is filled with the sample and a mixture of ampholytes capable of producing a pH gradient that covers the p*I* values of the proteins. Ampholytes are a mixture of hundreds to thousands of amphoteric compounds, generated by the random addition of acrylic acid to a mixture of linear and branched oligoamines, providing p*I* values are fairly well distributed along the pH scale from 3 to 10. In practice about 94% of proteins can be separated in the pH range 3–8.5. This allows a single capillary to be used for hundreds of separations by minimizing alteration to the capillary wall coating. When a voltage is applied (e.g. 15 kV for 4 min) the sample components focus into narrow zones according to their p*I* values. The zones are then mobilized by hydraulic, electroosmotic or ion addition (by adding 80 mM sodium chloride to either the source or destination vial and applying an electric field) to move them past the detector. The destination vial contains a buffer (catholyte) at a pH higher than the p*I* of the most basic ampholyte (40 mM sodium hydroxide) and the source vial contains a buffer (anolyte) at a pH lower than the p*I* of the most acidic ampholyte (20 mM phosphoric acid). To avoid protein precipitation in the focused zones a surfactant or urea can be added to the buffer, the sample diluted, or gel-filled capillaries can be used.

In capillary isotachophoresis sample ions are separated by differences in their mobility in a heterogeneous buffer system created by sandwiching the sample between a leading and terminating buffer with different and specified compositions. It is general practice to separate mixtures in the constant current mode using chemically bonded or dynamically coated capillaries to eliminate electroosmotic flow. As well as fused silica capillaries of standard dimensions wide-bore Teflon (0.5–0.8 mm) tubes have been used in purpose-built apparatus for isotachophoresis. Before commencing the separation both the capillary and destination buffer vial is filled with the leading electrolyte (assuming suppression of the electroosmotic flow). The leading electrolyte ion must have a higher mobility than any of the analytes to be separated and the counterion must be able to set the pH for the separation by ensuring sufficient (but generally not complete) dissociation of weak acids and bases in their own zones. Either sample cations or anions can be determined in the separation but not both simultaneously. The terminating electrolyte is placed in the source vial and should have a lower mobility than any of the analyte ions. Recommendations for buffer selection and operating conditions are summarized in **Table 8**. If solubility is a problem nonionic or zwitterionic surfactants or urea can be added to both the leading electrolyte and the sample. When fused silica capillaries are used hydroxypropylmethylcellulose, polyethylene glycol or polyvinyl alcohol can be added to the buffers to suppress electroosmotic flow through dynamic coating of the column wall. Detection of

Table 8 Composition of some common capillary isotachophoresis buffers*

Property	*pH*				
	2.0	3.3	4.5	6.0	8.8
Separation	Cations	Anions	Cations	Anions	Anions
Leading ion	10 mM HCl	10 mM HCl	10 mM KOAc	10 mM HCl	10 mM HCl
Leading counterion		β-Alanine	HOAc	Histidine	Ammediol
Leading additive		0.2% HPMC		0.2% HPMC	0.2% HPMC
Terminating ion	10 mM TRIS	10 mM caproic acid	10 mM HOAc	10 mM MES	10 mM β-Alanine
Terminating counterion	HCl			TRIS	$Ba(OH)_2$
Terminating pH	8.5			6.0	9.0
Recommendations					
	Cations			Anions	
Leading ion (20–30 mM)	K^+, NH_4^+, Na^+			Cl^-	
Terminating ion	H^+, or weak base (mobility $> H^+$)			OH^-, or weak acid (mobility $> OH^-$)	
Terminating counterion	Weak acid, $pK = pH_L \pm 0.5$			Weak base, $pK = pH_L \pm 0.5$	
Typical counterions	pH_L				pH_L
Formate	3.2–4.2			β-Alanine	3.1–4.1
Acetate	4.2–5.2			Histidine	5.5–6.5
MES	5.7–6.7			Imidazole	6.6–7.6
Glycine	9.1–10.1			TRIS	7.6–8.6
				Ethanolamine	9.0–10.0

See Table 4 for buffer abbreviations; Ammediol = 2-amino-2-methyl-1,3-propanediol; HPMC = hydroxypropylmethylcellulose; and OAc = acetate.

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the separated zones is usually by conductivity or UV-visible absorption. The method has high peak capacity since separated zone boundaries are sharp and close to each other to maintain continuity of the current. When the experimental conditions are correct a steady state is reached in which all zones are migrating at the same speed and the detector output is a series of steps, the length of which corresponds to the concentration of the ion. At first sight the data presentation may be confusing and this combined with the complex method development has suppressed interest in capillary isotachophoresis in favour of other chromatographic methods. The compelling advantage of isotachophoresis is its ability to trace enriched dilute samples, by 100-fold or more, and as a preconcentration or preseparation technique for capillary zone electrophoresis it is enjoying something of a renaissance.

Conclusions

The capillary electrophoretic methods are sufficiently established to ensure their continued laboratory use but not so mature that significant developments are unexpected in the near future. These developments are likely to be application driven and will impact on the method development process. New systems for separation of biopolymers using gels and entangled polymers, a wider range of surfactants for selectivity optimization in micellar electrokinetic chromatography, and tailor-made sorbents for selectivity optimization and control of electroosmotic flow in electrochromatography are just some expected improvements. Better models for predicting sample migration should aid computer-aided method development strategies and experimental design approaches for multiparameter optimization of complex mixtures should grow in popularity.

Further Reading

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ESSENTIAL GUIDES TO METHOD DEVELOPMENT IN EXTRACTION

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Introduction

Samples for extraction can be broadly categorized as solid, liquid or gaseous matrices. It is obvious that the different methods of extraction of analytes from these matrices will also vary. This guide provides an overview of the different approaches for extraction of analytes from these different matrices.

It is important to consider that extraction is only one part of the sample preparation protocol. Other steps are highlighted in **Figure 1**. A typical solid sample is most likely to be heterogeneous. This is a problem in the analysis, if appropriate steps have not been taken to remove a representative sample using a statistical approach. Failure to do so can make any subsequent extraction and analysis results meaningless.

Also of relevance to any subsequent extraction and analysis is whether the sample has been stored (and preserved, if necessary) in the appropriate manner to prevent losses of the analyte due to degradation and/or adsorption. It is necessary to consider, in the

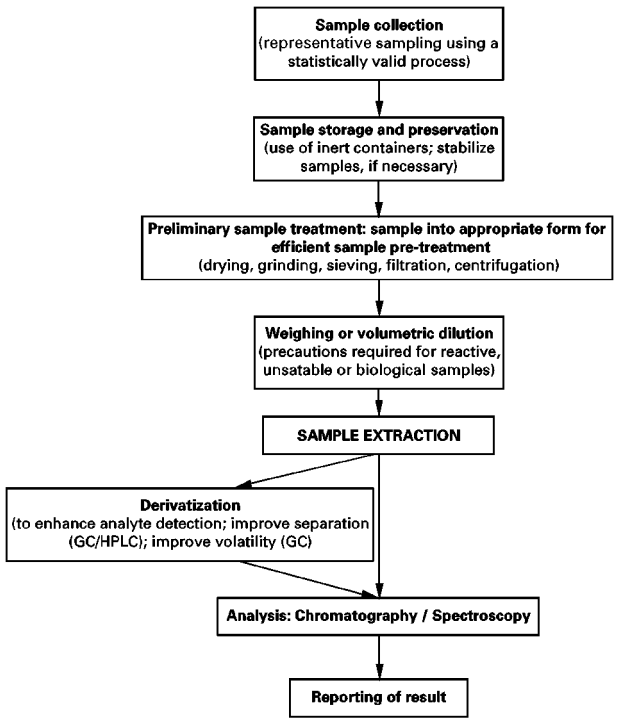
Sample collection
(representative sampling using a statistically valid process)
Sample storage and preservation
(use of inert containers; stabilize samples, if necessary)
Preliminary sample treatment: sample into appropriate form for efficient sample pre-treatment
(drying, grinding, sieving, filtration, centrifugation)
Weighing or volumetric dilution
(precautions required for reactive, unsatable or biological samples)
SAMPLE EXTRACTION
Derivatization
(to enhance analyte detection; improve separation (GC/HPLC); improve volatility (GC)
Analysis: Chromatography / Spectroscopy
Reporting of result

Figure 1 Sample preparation protocol.

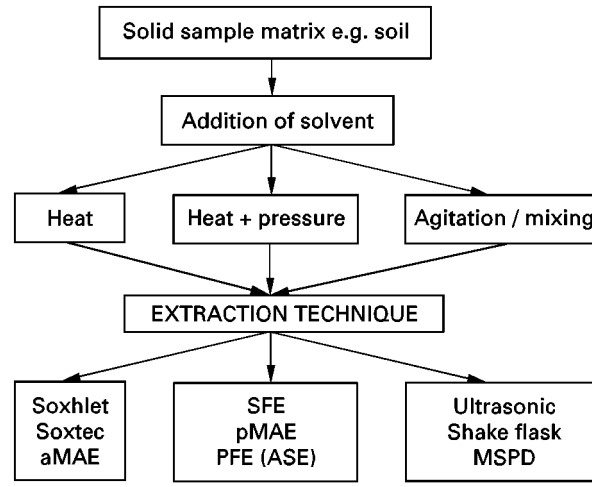

Figure 2 Extraction of analytes from solid matrices.